THE EFFECT OF LACK OF OXYGEN ON THE SPERM AND UNFERTILIZED EGGS OF *ARBACIA PUNCTULATA*, AND ON FERTILIZATION

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It has been shown in a former paper (Harvey, 1927) that when fertilized eggs are deprived of oxygen, development is arrested, and the eggs remain in whatever phase of division they were in when oxygen was taken away; they gradually resume development and pass through subsequent phases of division when oxygen is readmitted. The experiments were performed on two species of sea-urchin occurring at Naples, *Strongylocentrotus* (*Paracentrotus*) *lividus* and *Echinus microtuberculatus*. Some of these experiments have been repeated on the Woods Hole species, *Arbacia punctulata,* and have given the same results. The present paper deals with the effect of lack of oxygen on the *unfertilized* eggs and the sperm of *Arbacia punctulata,* and on the fertilization process in these eggs. The work was done during the summer of 1929 at the Marine Biological Laboratory of Woods Hole. I wish to thank the Director for the facilities of the laboratory.

The experiments on unfertilized eggs and sperm were carried out for the most part by bubbling hydrogen through a suspension of eggs or sperm in sea-water in a closed glass vessel, from which they could be drawn off at desired intervals for observation. The connection between the hydrogen tank and the glass vessel included a quartz tube containing platinized asbestos which was kept heated to redness to remove the last traces of oxygen; from here to the glass vessel, the connection was entirely of metal and glass, sealed with De Khotinsky cement, to avoid the leakage of air which takes place through rubber connections. The length of time for complete removal of air and replacement by hydrogen, of course, depends on size of vessel, amount of sea-water, rate of bubbling, etc., but under the conditions of the experiments it required approximately twenty minutes. That a state of complete anaerobiosis obtained was shown by the fact that under similar conditions the luminescence of luminous bacteria was stopped, as ascertained by E. N. Harvey.

When unfertilized eggs are thus kept without oxygen, they are very

little affected. During a period of exposure of 8 hours, one can observe no difference in appearance between the eggs when drawn from the hydrogen chamber and the control unfertilized eggs; and the exposed eggs can be fertilized and develop normally. For the first 3 hours, the eggs when withdrawn from the hydrogen chamber can be fertilized with as much ease and as rapidly as eggs kept in air; the fertilization membrane comes off at the same time (1–2 minutes) and the first cleavage plane comes in at exactly the same time (about 50 minutes) as in the control lots. When, however, eggs which have been exposed over 3 hours to hydrogen are withdrawn and fertilized, there is a slight lag (½–2 ½ minutes) in the formation of the fertilization membrane, a tendency of the membrane to adhere to the egg, a slight crenulation of the egg surface, and a lag of from 2 to 5 minutes in occurrence of the first cleavage. This was not due to the bubbling, for when air in place of hydrogen was bubbled for the same length of time through the same amount and concentration of eggs, these eggs when fertilized showed no lag in the formation of the fertilization membrane nor in time of cleavage over eggs kept at the same time undisturbed in watch glasses and fertilized. When eggs, which have been kept in hydrogen for three or more hours, are withdrawn and left in air unfertilized for 45 minutes and are then fertilized, they show no lag in membrane formation or in time of cleavage. The lag evidently represents the recovery time from exposure to the oxygen-free atmosphere. The unfertilized eggs have therefore a very short recovery period after a prolonged exposure to hydrogen, and recover instantly after a shorter exposure. They are thus in marked contrast to fertilized eggs, which require a comparatively long period (½ hour to 1 hour) for recovery from exposure to hydrogen before resuming development. It may be that the longer recovery period of the fertilized eggs from the effects of lack of oxygen is related to their greater oxygen consumption as compared with that of the unfertilized eggs. After exposure for 6 or 8 hours to either hydrogen or air (in the apparatus used) some of the eggs become cytolyzed, owing probably to the mechanical disturbance of the bubbling; the effect increases with time until, after about ten hours, practically all the eggs are cytolyzed. Whether, therefore, the life of the unfertilized egg is prolonged by lack of oxygen could not be determined by these experiments. Loeb and Lewis (1902) found that unfertilized eggs would live somewhat longer in absence of oxygen (64 hours) than in air (48 hours), and very much longer in a weak concentration (N/1000) of KCN (112 hours). This latter effect may, however, be due to destruction of harmful bacteria by the KCN as pointed out by Gorham and Tower (1902).

For experiments on sperm cells of *Arbacia,* a fairly concentrated suspension was used, one drop of fresh undiluted sperm to 10 cc. of sea-water (*i.e.,* about .6 per cent), giving a decidedly milky appearance. In such a concentration sperm live longer and retain their fertilizing power for a longer time than in a more dilute suspension, probably owing to CO_2 production as shown by Cohn (1918). When hydrogen is bubbled through the sperm suspension for about two hours, the sperm are motile immediately on withdrawal from the hydrogen chamber, or at least as quickly as they can be observed under the microscope. The lots of eggs into which they are immediately drawn form fertilization membranes and cleave at the same time as the controls. After an exposure of 2 to 3 hours, the sperm recover motility within a few seconds and fertilize eggs with a very slight lag over the controls. After an exposure of more than 3 hours, some of the sperm do not recover motility and only a fraction of the eggs to which they are added are fertilized. After 4 hours, the sperm are all inactive, do not fertilize the eggs and never recover. A control experiment in which air in place of hydrogen was bubbled through a similar amount and concentration of sperm showed that the deleterious effect is due to lack of oxygen and not to the mechanical agitation, since these sperm were just as active and potent for fertilization even after 9 hours of bubbling as are fresh sperm. It is interesting to note that the prevention of oxidations by means of a hydrogen atmosphere gives a different result from that obtained by the use of cyanides. Drzewina and Bohn (1912) found that the sperm of *Stronglyocentrotus* would survive and remain potent for 48 hours in KCN (1 : 1,000,000), and that when they were subjected to KCN for long periods (1 to 10 hours) they caused a more normal development of eggs than when subjected for a short period (30 minutes to 1 hour). Cohn (1918) found that KCN rendered *Arbacia* sperm inactive and prolonged their life, and in fact suggested that " whatever decreases the activity increases the length of their life." This is certainly not true for hydrogen. It may be, however, that some other factor associated with the absence of oxygen, such as the lack also of CO_2 is responsible for the death of the sperm in my experiments.

A study was made of individual sperm cells in the absence of oxygen by using a modified Engelmann chamber to which hydrogen was admitted and the sperm kept in a hanging drop (see Harvey, 1927). It was found that in many cases enough oxygen leaked through the vaseline seal with which the cover was mounted on the chamber to enable the sperm to keep their motility for several hours. By entangling the sperm in platinized asbestos threads, it was possible in some cases to keep

them absolutely oxygen-free, and they became motionless within a half hour. If air was then admitted, the sperm immediately became motile. Even if the bubbling of hydrogen was stopped, within a very few minutes the sperm became active. It apparently requires a very minute amount of oxygen for motility of the sperm. When sperm are kept in an Engelmann chamber without oxygen for two hours, they do not recover motility on admission of air. They are killed by the absence of oxygen even more quickly than when the experiments are done in bulk.

The most interesting question in connection with lack of oxygen on eggs and sperm is whether fertilization can take place and the fertilization membrane be thrown off during complete absence of oxygen. An attempt to answer the question was made by keeping unfertilized eggs in one drop and sperm in another drop very close together in an Engelmann chamber. Hydrogen was sent through for a half hour, then the chamber was shaken so as to make the drops coalesce and the sperm come in contact with the eggs, still keeping hydrogen passing through the chamber and the seal intact. It was found that when the sperm are completely immotile, they do not fertilize the eggs, probably because they cannot get to the surface of the egg; they go in currents around and past the eggs; in no case is a fertilization membrane thrown off. On admission of air the sperm become motile and the membranes of the eggs are thrown off in 1 to 2 minutes as normally. If there is the slightest trace of air leaking in the chamber, sufficient for a few only of the sperm to be very slightly motile, some of the eggs are fertilized on mixing the drops, and fertilization membranes are thrown off, but no further development takes place until more air is admitted. The question, therefore, whether oxygen is necessary for membrane formation has not been answered. If there is absolutely no oxygen, the sperm are absolutely immotile and cannot fertilize the eggs, probably owing to mechanical difficulties, and no membranes are given off. Loeb also found that if the sperm cells of *Strongylocentrotus* were made immotile by NaCN, they were unable to fertilize the eggs even when squirted on eggs with jelly removed. If in my experiments, there is the slightest trace of oxygen, a few sperm remain motile and fertilize eggs which throw off membranes. If membrane formation does require oxygen, it is in an almost infinitesimal amount. It requires more oxygen for the development of fertilized eggs than it does for motility of sperm, fertilization of the egg and the formation of the fertilization membrane.

Summary

1. Unfertilized eggs of *Arbacia* are not visibly affected by complete lack of oxygen for a period of 8 hours. After an exposure of 3 hours they recover immediately on admission of air; after a longer exposure, when air is readmitted and the eggs are fertilized, there is a slight lag in the formation of the fertilization membrane and in time of cleavage.

2. Sperm of *Arbacia* are rendered motionless by lack of oxygen, but are otherwise unaffected for 2 hours. They recover immediately on admission of air. After 3 hours some of the sperm are irreversibly injured, and after 4 hours they are all killed.

3. When sperm are added to unfertilized eggs, both being in complete absence of oxygen, fertilization does not take place, and the fertilization membrane is not thrown off because the sperm are not motile, and cannot get to the surface of the egg. The membrane is thrown off immediately on admission of air. If there is the slightest trace of air, which may leak through the vaseline seal to the chamber, sufficient for only a few sperm to be very slightly motile, the eggs with which they come in contact throw off fertilization membranes, but do not develop further until more air is admitted. If oxygen is necessary for membrane formation, it is in an almost infinitesimal amount.

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